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ORIGINAL ARTICLE

# Expression of key components of the RNAi machinery are suppressed in *Apis mellifera* that suffer a high virus infection

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**Abstract**

Viruses are one of the major threats for honeybees and until now more than 20 different viruses have been discovered. Viruses and their hosts are engaged in a continuous arms race in which viral defense mechanisms drive the adaptive evolution of host immune genes, which in turn results in counter-adaptations of the viral immune antagonists. The honeybee immune responses to non-viral pathogens have been extensively studied, but little is known about the antiviral responses. Recent evidence suggests that the main mechanism of antiviral defense in insects is the RNA interference (RNAi) pathway. Furthermore, there is evidence that some viruses suppress this RNAi pathway in order to evade antiviral immunity. In the present study, we test this hypothesis by comparing the gene expression levels of some key components of the RNAi response of honeybees that were naturally infected with at least five viruses with those who were infected with only two or three viruses using a colorimetric microarray developed in-house, called BeeClinic, and subsequently confirmed by quantitative reverse-transcription–polymerase chain reaction (qRT-PCR). Our results show that key components of the RNAi pathway are indeed downregulated in highly infected bees. We were able to show that high virus loads suppress key RNAi components, which results in a counteraction of the host RNAi antiviral defense. As the RNAi is a primary defense against viruses, these findings shed new light on pathogen–host interactions and can help mitigate escalating colony losses worldwide.

**Key words:** antiviral, BeeClinic, colorimetric microarray, Dicer, gene expression, honeybee.

## INTRODUCTION

Honeybees are exposed to a wide array of viruses (Chen & Siede 2007; Runckel *et al.* 2011; Li *et al.* 2014; McMenamin & Genersch 2015). Like all insects they lack an adaptive immune system and thus, the immune defense relies solely on the innate immune response, which is based on a constitutively active cellular and an inducible humoral immune response (Evans *et al.* 2006). Based on the honeybee genome, homologue members of the complex humoral immune response could be identified (Evans *et al.* 2006). Insects are able to trigger various defense pathways depending on the type of infecting pathogen, and most of these pathways are interconnected. For fungal and bacterial infections, the Toll, Imd and Jak/STAT pathways have been implicated (Lemaitre & Hoffmann 2007). Although these pathways also play a role in the clearance of viral infections their antiviral function seems to be virus-specific rather than being a generic antiviral response (Kemp & Imler 2009). The interaction of the virus with the host's innate immune system plays a critical role in the outcome of the infection. The major mechanism of antiviral defense is the RNA interference (RNAi) pathway (Ding 2010). Most honeybee viruses are positive-sense single-stranded RNA (+ssRNA) viruses, which produce double-stranded RNA (dsRNA) replication intermediates (Chen 2011). This virus-generated dsRNA serves as a template for the RNAi machinery and produces small, interfering RNAs (siRNAs) that function to target viral RNA for degradation and hence inhibit replication (Ding 2010). Direct evidence of the antiviral role of RNAi insects comes

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from studies in *Drosophila melanogaster*, *Aedes aegypti* and *Anopheles gambiae* (Campbell *et al.* 2008; Saleh *et al.* 2009; Blair 2011). Recent studies in *Apis mellifera* and *Apis cerana* demonstrated the role of RNAi antiviral immunity in honeybees (Maori *et al.* 2009; Liu *et al.* 2010; Desai *et al.* 2012). In general, RNAi-based degradation of dsRNA involves the production of small non-coding RNAs, and their biogenesis and function are based on two proteins: Dicer (Dcr) and Argonaute (Ago) (Elbashir *et al.* 2001; Hammond *et al.* 2001). Their genes are strongly conserved in a wide range of species. However, as a result of evolutionary and immune adaptation processes, there are several paralogues of both proteins. The RNAi response is conserved in every insect model tested until now (Karlikow *et al.* 2014). The role of the RNAi response as an immune system combating viruses in bees is scarcely studied although recently its involvement in bumblebees has been shown (Niu *et al.* 2015; Piot *et al.* 2015). The inducer signal for a systemic response remains unknown. Nevertheless, evidence points to a signal of RNA nature (dsRNA, small RNA or RNP).

The honeybee immune responses to non-viral pathogens have been extensively studied (Chan *et al.* 2009; Schwarz & Evans 2013), but far less is known about the immune system's antiviral responses. Recently, it has been shown that workers from collapsed honeybee colonies display an RNAi response. High-throughput sequencing and data analysis of small RNA from these bees showed a high number of reads of small RNA of 21–22 nt perfectly matching the Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV) and deformed wing virus (DWV) genomes in these colonies (Flenniken & Andino 2013; Chejanovsky *et al.* 2014).

RNAi has also been associated with controlling the persistence of RNA virus infections in *Drosophila* (Goic *et al.* 2013). In honeybees, severe *Varroa*-mite infestations are often associated with persistent DWV infection (de Miranda *et al.* 2012). This hematophagous mite acts as vector for many viruses, but overt DWV infections in the pupal stage are very common. In such pupae, virus-specific siRNAs (mostly derived from DWV and 22 nt in length) could be identified, but the virus-specific siRNA levels were not always proportional to the level of viral genomic RNA (Ryabov *et al.* 2014). This supports the hypothesis that suppression of the antiviral response may also exist. The detection of virus-specific siRNAs in several studies suggests a normal functioning of Dicer in honeybees (Desai *et al.* 2012). However, transcriptome studies studying the honeybee response to viral infections could not show significant changes in gene expression of the key components of the RNAi response, such as *dcr* and *ago* (Ryabov *et al.* 2014). In *Drosophila*, cricket paralysis virus (CrPV) has been found to encode a potent suppressor that mutes the RNAi antiviral response (Nayak *et al.* 2010). This suppressor is located upstream of a highly conserved sequence (DVEXNPGP) within the N-terminal regions of CrPV open reading frame-1 (ORF-1) (Bennasser *et al.* 2006). This conserved motif (DIEENPGP) is also identified in the N-terminal region of ORF-1 of IAPV and other members of the Dicistroviridae family infecting honeybees such as KBV and acute bee paralysis virus (ABPV) (Chen *et al.* 2014). Silencing of this putative immune suppressive protein led to significant reduction in IAPV replication, which suggests that IAPV may encode an RNAi suppressor (Chen *et al.* 2014). Viral suppressors of RNAi (VSR) proteins are often encoded in overlapping reading frames or suppressor activity will have evolved in unrelated viral proteins with other functions (Li & Ding 2006). As a result, viral suppressors differ greatly with respect to sequence, structure, and mode of action. The best studied VSR proteins are RNA-binding proteins that shield dsRNA produced during viral infection from Dicer processing and RNA-induced silencing complex (RISC) assembly (O'Neal *et al.* 2014). Other VSR proteins do not bind dsRNA or siRNAs, but exert an inhibitory effect on RNA silencing through a direct interaction with *Ago2* (O'Neal *et al.* 2014). It was also shown that Dicer, Drosha and *Ago2* mRNA and protein expression levels were downregulated in mammalian cells infected with influenza A virus, dengue virus and hepatitis B, which suggests that Drosha, Dicer and *Ago2* regulate viral replication (Chinnappan *et al.* 2014). In this study, we compared the gene expression levels of some key components of the RNAi response of naturally infected honeybees using a colorimetric microarray fabricated in-house. In particular, we compared the expression level of bees that were naturally infected with at least five viruses with those that were infected with two or three viruses. Our prediction was that that the RNAi response would change with a higher virus load.

## MATERIALS AND METHODS

### Honeybee samples

Honeybee samples with low and high virus loads were selected from previous studies (De Smet *et al.* 2012; Ravoet *et al.* 2013). All the bees were sampled at the bee hive entrance, which means that they are at least four weeks old. Bees with a low virus load were infected by two or three viruses while those with a high virus load were infected with at least five viruses. Deformed wing virus and bee macula like virus were retrieved in almost all samples. The exact virus load for the different selected samples is given in Table S1. Eight colonies for each

condition were selected, from which three bees were analyzed using the BeeClinic microarray. For the quantitative reverse-transcription–polymerase chain reaction (qRT-PCR) validation experiment the same bee samples were used.

## Preparation of total RNA

Total RNA was isolated using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) starting from one complete honeybee. The tissues were homogenized by mechanical agitation in a TissueLyser (Precellys, PEQLAB Biotechnology, Germany) for 90 s at 30 Hz in the presence of a pair stainless steel beads and 1 mL Qiazol (Qiagen) lysis reagent. The total RNA was isolated according to the recommendations of the manufacturer's protocol, eluting the RNA in a final volume of 50 µL. The concentration of the total RNA was measured using a Nanodrop (Isogen; Nippon Gene, Tokyo, Japan).

## Design of colorimetric microarray

The principle of the colorimetric hybridization assay is outlined in Figure 1. The sample, which contains digoxigenin (DIG)-labeled cDNA, is used for hybridization. The hybridization signals are developed using anti-DIG antibodies (Roche Applied Science, Indianapolis, IN, USA), which are alkaline phosphatase (AP) conjugated. The AP system enables a colorimetric detection of hybridization: the immobilized enzyme's reaction with its substrate (BCIP/NBT) generates a purple precipitate on hybridized spots. The stained microarrays are finally imaged using a high-resolution flatbed scanner and the grayscale image is analyzed.

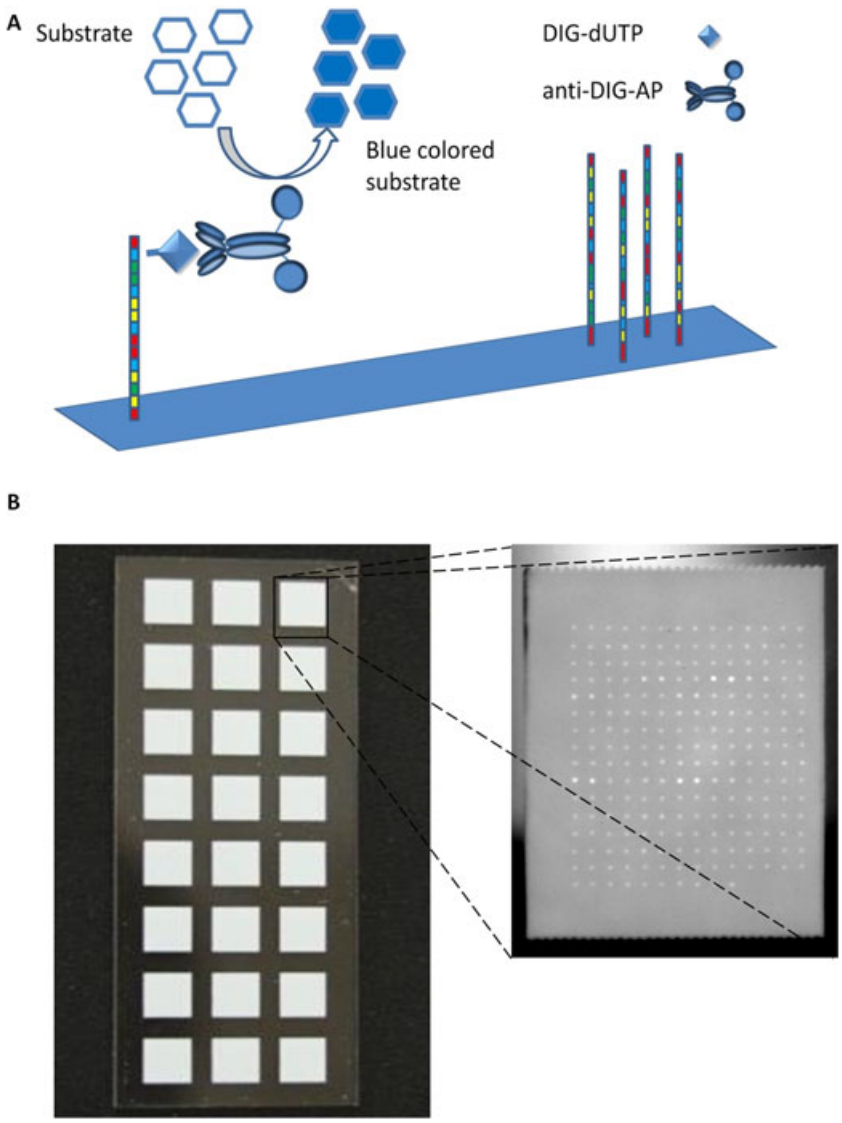


**Figure 1** (A) General outline of the in-house microarray procedure: 110 targets are printed per microarray. After hybridization of the digoxigenin (DIG)-labeled cDNA, an anti-digoxigenin antibody-alkaline phosphatase conjugate is bound to the hybridized probe. The signal is detected with the colorimetric alkaline phosphatase substrate, NBT and BCIP. (B) 24-plex microarray slide and close-up of one field after development.

## Targeted genes

Our array targeted immunity genes that were previously included in the Beepath quantitative PCR array (Evans 2006) and was further extended with selected pathogen probes and markers for *Varroa* infestation (Alaux *et al.* 2011), *Nosema* infection (Dussaubat *et al.* 2012), nutritional stress (Alaux *et al.* 2011) and genes involved in ageing and in the RNAi machinery. For some threats, genome-wide transcriptome studies were available (Alaux *et al.* 2011; Dussaubat *et al.* 2012). For each threat, the five most up- and downregulated marker genes were selected from these studies. Based on quantitative trait locus (QTL) analysis, two genes for *Varroa* resistance (Behrens *et al.* 2011) and one gene for *Nosema* resistance (Huang *et al.* 2012) were also selected and included in the target list. Genes implied in ageing or in the RNAi machinery were selected based on literature data and homology (reviewed by Karlikow *et al.* 2014) with known *Drosophila* genes involved in the RNAi response (McMenamin & Genersch 2015). This resulted in a total final set of 109 targets included on our array (Table S2). The corresponding oligonucleotides from the selected targets were used from the study of Johnson *et al.* (2009). New oligonucleotides were designed using the AlleleID7 (Premier Biosoft International; Palo Alto, CA, USA). All the probes were synthesized and desalted by Integrated DNA Technologies (Coralville, IA, USA). The probes were printed in duplicate on nitrocellulose coated glass slides by ArrayIT (Sunnyvale, CA, USA). Each slide is composed of 24 identical arrays, with each array harboring 109 targets in duplicate. The printed arrays were stored at room temperature until use.

## Labeling cDNA

The cDNA was labeled with DIG using the Superscript Direct cDNA labeling system from Invitrogen (Carlsbad, CA, USA). Briefly, 25 μg of total RNA was reverse transcribed into cDNA with anchored oligo(dT)20 primers. The reverse transcription procedure was used as described by the manufacturer, except that deoxynucleoside triphosphate (dNTP) was replaced by 2 mM dATP, dGTP and dCTP, 1.3 mM dTTP and 0.7 mM alkali stable DIG-dUTP (Roche). Once the reverse transcription was completed, the enzyme was inactivated and the original RNA was degraded by alkaline hydrolysis. The labeled cDNA was purified using the Superscript III Direct Purification Module (Invitrogen) according to the kit instructions. Purified single-strand cDNA was eluted from the columns in 70 μL DEPC-treated water.

## Hybridization and development

Before hybridization, the microarrays are washed three times in BlockIT solution (ArrayIT) to remove unbound nucleotides, followed by a prehybridization step with BlockIT solution for 1 h at room temperature. After blocking, the array was washed three times for 5 min with 5×SSC (0.15 M NaCl, 0.015 M sodium citrate for 1× SSC) and 0.1% sodium dodecyl sulfate and mounted in a 24-well hybridization cassette from ArrayIT. Labeled cDNA corresponding with 3 μg total RNA was mixed with hybridization buffer (final concentration 25% formamide, 5× SSC and 0.1% SDS) to a final volume of 75 μL and heated for 1 min at 65°C to denature the cDNA and snap-cooled on ice for 30 s. The labeled cDNA was then transferred to the array and hybridized overnight at 33.5°C. After hybridization, the arrays were unmounted from the cassette and washed twice at 45°C for 5 min with 2× SSC and 0.1% SDS, twice with 0.5× SSC and 0.1% SDS at 45°C for 5 min followed by a 5 min wash at room temperature with 0.5× SSC.

Colorimetric detection is a three-step process. In the first step, membranes were treated with 1% blocking solution (Roche) for 30 min to prevent nonspecific attraction of the antibody to the membrane. In the following step the membranes were incubated with 1500× diluted anti-digoxigenin (Roche) in 1% blocking solution. The antibodies are conjugated to alkaline phosphatase, which made colorimetric development possible. The unbound antibody was washed away in two washing steps for 15 min with PBS and the slides were equilibrated in TBS buffer for 5 min. In the last step, the membrane carrying the hybridized probed and bound antibody conjugate was reacted with the colorimetric detection reagents nitro blue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The NBT/BCIP stock solution (Roche) contained 18.75 mg/mL nitro blue tetrazolium chloride and 9.4 mg/mL BCIP, toluidine-salt in 67% DMSO (v/v). The slides were incubated in 15 mL TBS buffer containing 300 μL NBT/BCIP for 30 min. The reaction was stopped by washing the slides in distilled water. The array was dried by centrifugation. Subsequently, the slides were scanned using the ArrayIT SpotWareTM colorimetric microarray scanner at 16-bit grayscale depth and 5 μm resolution and saved in TIFF format.

## Data analysis

The TIFF images were processed with Mapix (Innopsys, France) to ascribe a value to the spot intensity, which was corrected by background intensity. The intensity data were standardized across different samples using the

control reference genes *RPL8* and *actin*. To test for differential expression, the Bayesian adjusted *t*-statistics from the linear models for Micoarray data (limma) package were used (Smyth 2004; Ritchie *et al.* 2015). The adjusted *P*-value was calculated using the method developed by Benjamin and Hochberg (Evans & Spivak 2010).

### Validation by qRT-PCR

Using random hexamer primers, 2 μg total RNA was retro-transcribed with the RevertAid H Minus First Strand cDNA Synthesis Kit (Invitrogen). Expression levels of genes involved in the RNAi pathway were quantified by qPCR to confirm the results from the colorimetric microarray analysis. Primers for 11 reference genes and some target genes (Table S3) were used from the literature or newly designed with Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the default settings. For the RT-qPCR assays the SsoFast EvaGreen Supermix kit (Bio-Rad, Richmond, CA, USA) was used. Each 15 μL reaction consisted of 7.5 μL master mix, 0.2 μM forward and 0.2 μM reverse primers (Integrated DNA Technologies) and 1 μL cDNA template using the CFX96 Real-Time PCR Detection System (Bio-Rad). The PCR program comprises an activation step of 1 min at 95°C and 40 cycles of a combined denaturation (5 s at 95°C) and annealing (10 s at 60°C) step. At the end of this program a melt curve is generated by measuring fluorescence after each temperature increase of 0.5°C for 5 s over a range from 65°C to 95°C. Primer efficiencies, R2 values and melt curves were calculated with CFX Manager software (Bio-Rad). Reference gene stability was analyzed with the geNormPLUS algorithm within the qBasePLUS environment (Biogazelle NV, Zwijnaarde, Belgium) with default settings. Differential gene expression of target genes was statistically analyzed using qBasePLUS by means of Mann–Whitney tests. Two-sided significance and correction for multiple testing were applied.

## RESULTS

### Overview of differential gene expression from some target genes as revealed by microarray analysis

The effect of natural viral infection on the gene expression of the different targets was determined by comparing the gene expression of naturally high virus infected (HVI) honeybees with that of low virus infected (LVI) honeybees. The expression levels were normalized using *RPL8* as a reference gene. In Table 1, the expression ratios from different immunity genes and genes involved in the RNAi antiviral response from HVI and LVI honeybees are given. As the dynamic range of the colorimetric array was low, we used this array as a first screening method to determine a set of differential expressed genes. Most of the immunity genes in the HVI individuals were diminished in expression levels compared to LVI individuals. The gene expression levels of most of the genes involved in the RNAi machinery were also decreased in the HVI group. The results of the screening with the micro-array, developed in-house and called BeeClinic, showed that high virus loads in honeybee are accompanied by suppression of the key components of the RNAi machinery.

### Reference gene selection for normalization of qRT-PCR data

The reliability of the data of the microarray experiment was checked with qRT-PCR. To correct for experimental error, qPCR data require normalization against reference genes (Bustin *et al.* 2009). Thus, 11 reference genes were selected (Table S3) and their expression was quantified in HVI and LVI honeybees. The genes from most to least stably expressed across all conditions produced the following ranking (Fig. S1A): *eIF* > *RPS5* > *RPL8* > enolase > *MGST* > *GAPDH* > actine > *RP49* > *TBP* > *RPL13a* > *RPS18*. Another measure, the geNorm V-value, is useful for determining the optimal number of reference genes for data normalization (Fig. S1B): V2/3 (0.214) – V3/4 (0.157) – V4/5 (0.200) – V5/6 (0.166) – V6/7 (0.155) – V7/8 (0.151) – V8/9 (0.141). Setting the threshold to 0.15, six genes should be included in the calculation of the normalization factors (Vandesompele *et al.* 2002; Hellemans *et al.* 2007).

### Validation of microarray data by qRT-PCR

Microarray validation by qRT-PCR was performed with all genes involved in the RNAi machinery (Table 2). For four genes, Dicer-like (*Dcr-like*), Dicer1 (*Dcr1*), scavenger receptor class C type I (*SCR-C*) and TAR RNA-binding protein (*TARBP*), we could show a significant decrease in expression in the HVI group. However, it is worthwhile to mention that all the other genes involved in the RNAi machinery showed also lower expression levels, which suggest a suppression of the entire RNAi machinery. Expression profiles obtained in the qRT-PCR experiment were similar to those of the microarray experiment, except that for *Ago2* opposite expression levels were obtained.

## DISCUSSION

Viruses are one of the major threats for honeybees and until now, more than 20 different viruses have been

**Table 1** Expression ratios from different immunity genes and genes involved in the RNAi anti-viral response from HVI versus LVI honeybees

| ID | Expression change | logFC | *P* | adj. *P* |
|---|---|---|---|---|
| Immunity-related genes | | | | |
| *Myd88* | ↓ | –0.2138400696 | 0.0162492275 | 0.3668728645 |
| Defensin2 | ↓ | –0.3123921013 | 0.1115784119 | 0.5895526928 |
| *Lys-2* | ↓ | –0.0956489282 | 0.1697480242 | 0.6378612144 |
| Apisimin | ↑ | 0.3539765077 | 0.2004993572 | 0.6501039765 |
| pGRP9710 | ↓ | –0.1345275462 | 0.2565079163 | 0.6619780418 |
| Apidaecin | ↑ | 0.1661452114 | 0.3721433519 | 0.7203635361 |
| Hopscotch | ↓ | –0.1498095232 | 0.3467253496 | 0.7203635361 |
| Kenny | ↓ | –0.1356421311 | 0.3535630254 | 0.7203635361 |
| pGRPLC710R | ↓ | –0.1127950494 | 0.3787671635 | 0.7203635361 |
| Basket | ↑ | 0.0713399039 | 0.4646187816 | 0.7757623218 |
| *BglucA* | ↓ | –0.1088962985 | 0.5147128215 | 0.7757623218 |
| Cactus-1 | ↓ | –0.1090418077 | 0.4819047884 | 0.7757623218 |
| Domeless | ↓ | –0.1319723889 | 0.5308397298 | 0.7757623218 |
| Dorsal-1 | ↓ | –0.0693316542 | 0.462003764 | 0.7757623218 |
| *Lys-1* | ↓ | –0.0498326995 | 0.500786236 | 0.7757623218 |
| Perseph | ↓ | –0.1270461796 | 0.4807258399 | 0.7757623218 |
| pGRPSC2505 | ↓ | –0.1153286919 | 0.4836842337 | 0.7757623218 |
| *PPOact* | ↓ | –0.1171048375 | 0.4843593599 | 0.7757623218 |
| Spaetzle | ↓ | –0.0780592746 | 0.5301151514 | 0.7757623218 |
| Hemipterous | ↓ | –0.1192836181 | 0.573967886 | 0.7788792605 |
| *Tab* | ↓ | –0.0622960477 | 0.5808615216 | 0.7788792605 |
| Dredd | ↓ | –0.0756838627 | 0.595243977 | 0.7863099449 |
| Cactus-2 | ↓ | –0.0620173169 | 0.6099163588 | 0.7912324918 |
| Relish | ↓ | –0.0470041476 | 0.6137597833 | 0.7912324918 |
| Dscam-37 | ↑ | 0.0466894594 | 0.6451372703 | 0.821454886 |
| *TEPA* | ↓ | –0.0411024341 | 0.7394139642 | 0.8790810463 |
| *Lys-3I* | ↓ | –0.041268914 | 0.800169192 | 0.9306315603 |
| Abaecin | ↑ | 0.0396080372 | 0.809465864 | 0.9313209403 |
| *Dscam* | ↓ | –0.0233648515 | 0.8244768753 | 0.938500273 |
| *EGFlikeA = Eater* | ↓ | –0.0235614594 | 0.8475223432 | 0.9530921315 |
| Defensin1 | ↑ | 0.0241697618 | 0.9136585636 | 0.961897805 |
| Hymenopt | ↑ | 0.0182766292 | 0.9158476679 | 0.961897805 |
| *Imd* | ↓ | –0.010126515 | 0.9208859191 | 0.961897805 |
| *pGRPSC4300* | ↓ | –0.0189983869 | 0.906768034 | 0.961897805 |
| *TEP7* | ↑ | 0.0257638135 | 0.8829159556 | 0.961897805 |
| *AmPPO* | ↑ | 0.0058257697 | 0.9646463432 | 0.9764964622 |
| Dorsal-2 | ↑ | 0.0078146459 | 0.9508312236 | 0.9764964622 |
| *Tak1* | ↑ | 0.0034580313 | 0.9673703271 | 0.9764964622 |
| *Toll* | ↓ | –0.0053407889 | 0.9790570823 | 0.9790570823 |
| RNAi-related genes | | | | |
| Aubergine | ↓ | –0.4750271686 | 0.0054629947 | 0.3668728645 |
| Dicer2 | ↓ | –0.301630163 | 0.0279449642 | 0.3858134763 |
| Dicer1 | ↓ | –0.2740781564 | 0.0503066365 | 0.4485675084 |
| *Ago2* | ↑ | 0.3869129667 | 0.1122432583 | 0.5895526928 |
| Dicer2 | ↓ | –0.3120556635 | 0.1154024703 | 0.5895526928 |
| *TRBP2* | ↓ | –0.2775895973 | 0.1183784066 | 0.5895526928 |
| *Sid-I* | ↓ | –0.3161457773 | 0.0781852908 | 0.5895526928 |
| *Ago1* | ↓ | –0.2063470201 | 0.1907622324 | 0.6378612144 |
| Pasha homologue | ↓ | –0.2466573824 | 0.1664867898 | 0.6378612144 |
| *SRC-C* | ↓ | –0.2321851438 | 0.1631458939 | 0.6378612144 |
| *TARBP* | ↓ | –0.1562478829 | 0.2411147007 | 0.6619780418 |
| Drosha homologue | ↓ | –0.1892093027 | 0.3266593987 | 0.7203635361 |
| Dicer2 | ↑ | 0.1182388252 | 0.374073057 | 0.7203635361 |

*(Continues)*

TABLE 1. *(Continued)*

| ID | Expression change | logFC | *P* | adj. *P* |
|---|---|---|---|---|
| *Ago3* | ↑ | 0.1257475242 | 0.4929145669 | 0.7757623218 |
| Argonaute2 | ↓ | –0.1030457037 | 0.4961182051 | 0.7757623218 |
| *Stau* | ↑ | 0.0183886426 | 0.9168821052 | 0.961897805 |
| Other marker genes | | | | |
| *mFor* | ↓ | –0.3955084084 | 0.0076561118 | 0.3668728645 |
| *IRS* | ↓ | –0.3000527674 | 0.0205723102 | 0.3668728645 |
| Juvenile hormone esterase | ↓ | –0.3813597363 | 0.0205536632 | 0.3668728645 |
| *TOR* | ↓ | –0.3346384267 | 0.0118364791 | 0.3668728645 |
| *InR-2 (daf-2)* | ↓ | –0.4080606105 | 0.0311102591 | 0.3858134763 |
| Glucose dehydrogenase | ↓ | –0.3341599151 | 0.0464487427 | 0.4485675084 |
| *Rpd3* | ↓ | –0.3391419852 | 0.0472851649 | 0.4485675084 |
| α Mannosidase I | ↓ | –0.2116531287 | 0.1267262798 | 0.5895526928 |
| Hemolectin | ↓ | –0.5304977143 | 0.0869008049 | 0.5895526928 |
| Malvolio | ↓ | –0.2652851109 | 0.0795292457 | 0.5895526928 |
| Painless | ↓ | –0.2699401084 | 0.1177320908 | 0.5895526928 |
| Sluggish A | ↓ | –0.2413689417 | 0.1071555905 | 0.5895526928 |
| *dSir2* | ↓ | –0.2532656245 | 0.1820991154 | 0.6378612144 |
| Pheromone biosynthesis-activating neuropeptide | ↑ | 0.1930591825 | 0.185453636 | 0.6378612144 |
| Stretchin-Mlck | ↓ | –0.2288870438 | 0.1904190464 | 0.6378612144 |
| Thioredoxin reductase-1 | ↓ | –0.1945863754 | 0.1697007671 | 0.6378612144 |
| Hexamerin 70a | ↓ | –0.1758682431 | 0.2087699864 | 0.6570114278 |
| Catalase | ↓ | –0.2030069205 | 0.2227440116 | 0.6605118885 |
| α glucosidase (Hbg1) | ↓ | –0.1345532464 | 0.2598418482 | 0.6619780418 |
| Hairy | ↓ | –0.1229296117 | 0.251853108 | 0.6619780418 |
| Armadillo | ↑ | 0.118474724 | 0.2729416256 | 0.6791803242 |
| Corticotropin releasing hormone binding protein | ↓ | –0.1295469453 | 0.3400031205 | 0.7203635361 |
| Cytochrome P450 monooxygenase (Cyp4g11) | ↑ | 0.104544683 | 0.3322755967 | 0.7203635361 |
| Facilitated trehalose transporter Tret1-like | ↓ | –0.1534007353 | 0.3492074685 | 0.7203635361 |
| Poly U binding factor 68kD | ↓ | –0.1272054412 | 0.3837450613 | 0.7203635361 |
| Prophenoloxidase | ↓ | –0.1244017237 | 0.3040654398 | 0.7203635361 |
| Sugarless | ↓ | –0.0964444261 | 0.3732255014 | 0.7203635361 |
| Futsch | ↓ | –0.100580448 | 0.5365085216 | 0.7757623218 |
| Superoxide dismutase | ↑ | 0.1092696452 | 0.5078646115 | 0.7757623218 |
| Trehalose transporter 1 | ↓ | –0.102442541 | 0.5266524472 | 0.7757623218 |
| *EGFR* | ↑ | 0.0646858736 | 0.582339634 | 0.7788792605 |
| Na pump subunit | ↓ | –0.0813559192 | 0.5605051046 | 0.7788792605 |
| Vitellogenin | ↓ | –0.1085209875 | 0.5725400384 | 0.7788792605 |
| Dopamine receptor, D1 | ↓ | –0.0731462832 | 0.6602347682 | 0.821454886 |
| dPGC-1/spargel | ↓ | –0.0694665547 | 0.6942751682 | 0.8346903708 |
| *Foxo* | ↑ | 0.0638067343 | 0.7700727751 | 0.9054701861 |
| Transferrin | ↑ | 0.0249853511 | 0.8551106974 | 0.9530921315 |

HVI, high virus infected; LVI, low virus infected; log FC, log (fold expression); adj. *P*, adjusted *P*-value calculated using the BH method (Smyth 2004); ↑, unregulated; ↓, downregulated.

discovered (Evans & Schwarz 2011). Viruses and their hosts are in continuous competition in which viral counter-deface mechanisms drive the adaptive evolution of host immune genes, which in turn results in counter-adaptations of the viral immune antagonists (Daugherty & Malik 2012).

Most honeybee viruses are +ssRNA viruses. These viruses produce dsRNA as replication intermediates. The dsRNA is often a molecular signature of virus infection and as such is a trigger for a range of host responses, including RNA interference. Viral dsRNA may feed into the RNAi machinery to restrict virus replication (Bronkhorst & van Rij 2014). Several mechanisms were predicted by which virus infections can interfere with RNAi (Swevers *et al.* 2013). These include the expression of VSRs, accumulation of large amounts of viral RNAs and small RNAs that will overflow the RNAi machinery, modulation of expression of host miRNAs and expression of viral miRNAs, and induction of a general antiviral state in infected insects.

**Table 2** Quantitative polymerase chain reaction validation results

| Gene | Ratio | *P*-value |
|---|---|---|
| *Ago* | 0.477 | 0.075 |
| *Ago2* | 0.160 | 0.094 |
| *Ago3* | 0.552 | 0.084 |
| *Aub* | 0.443 | 0.316 |
| Dicer2 | 0.264 | $4.615 \times 10^{-2}$* |
| Dicer1 | 0.529 | $4.615 \times 10^{-2}$* |
| Drosha homologue | 0.771 | 0.399 |
| *SCR-C* | 0.278 | $4.615 \times 10^{-2}$* |
| *TARBP* | 0.423 | $4.930 \times 10^{-2}$* |

* Significantly different.

Different studies in honeybees could already show the existence of virus-specific siRNA on viral infection but until now evidence is lacking about the exact mechanism (Flenniken & Andino 2013; Ryabov *et al.* 2014). Several transcriptome studies could not show significant changes in gene expression of key components, such as Dicer and Argonaute, of the RNAi response (Flenniken & Andino 2013; Ryabov *et al.* 2014). These studies were mostly performed with experimentally infected honeybees. The advantage of working with experimentally infected honeybees is that the genetic background of all the bees is the same. On the other hand, mostly just emerging bees are artificially infected, which means that the immunity response is determined on a relatively short period. When working with naturally infected honeybees the infections occurred naturally and the copy number of the different viruses will be relatively low; moreover bees benefit from social immune behaviors (i.e. grooming and behavioral fever), which may reduce colony pathogen burden (Evans & Spivak 2010) and will not be present in caged honeybees. Another advantage of working with naturally infected honeybees is that in this way the natural response of the honeybee in its natural environment can be studied. We selected natural HVI honeybees and performed an in-house colorimetric array where all key components of the RNAi response were targeted. Although the dynamic range of the colorimetric array is low and no significant gene expression could be shown, we were able to show changed expression levels of all genes involved in the RNAi machinery. This screening method is meant for a rapid screening of changed expression levels from a selected set of targets and show trends in the expression levels of a certain set of selected targets. The expression levels of most targets were lower in highly infected honeybees compared to LVI bees. Validation with qPCR showed that the genes encoding two Dicer proteins, SCR-C and TARBP, were expressed significantly lower. It seems that the viruses are able to highjack the RNAi machinery although the exact mechanism remains unclear. Emerging evidence indicates that some animal viruses alter the expression of components of the RNAi machinery. Dicer mRNA and protein expression levels were also downregulated in mammalian cells infected with influenza A virus or dengue virus (Matskevich & Moelling 2007). Dicer, TARBP and SCR-C are all proteins that can be targeted by VSRs and it has been shown that the mRNA levels can also be modulated through viral infection. Viral suppressors do not have common sequence motifs and act at different steps of the RNAi pathway. VSRs can interfere with the activity of Dicer, which leads to suppression of siRNA biogenesis (Qi *et al.* 2012). TARBP is a co-factor of Dicer that functions as part of the RISC complex and was shown to be a target of VSRs encoded by HIV-1 (Bennasser *et al.* 2006). The mRNA levels for both targets were significantly downregulated in our study, which suggests the existence of VSR expression in honeybee viruses. The mRNA levels of SCR-C were also significantly lower in highly infected honeybees. This is a receptor that is important for dsRNA uptake. In *Drosophila*, exogenous dsRNA enters the RNAi pathway by scavenger receptor-mediated endocytosis and it was shown that RNAi uptake is essential in the process of antiviral defense. However until now there is no evidence that SCR-C is a target for virus-encoded VSRs.

Our results suggest that honeybee viruses are able to usurp the host RNAi machinery by means of suppression genes of the key components. The recent discovery that honeybee viruses of the ABPV complex encode VSRs located upstream of a DVEXNPGP motif located in the N-terminal region of ORF-1 (Chen *et al.* 2014) strongly supports this hypothesis.

In conclusion, we analyzed the differential expressed genes in honeybees with a high virus load compared to honeybees with a low virus load. The microarray developed in-house, called BeeClinic, showed that the genes involved in the RNAi pathway are mostly suppressed, which was confirmed by qRT-PCR. The suppression of the different key RNAi components (*Dcr2*, *SCR-C* and *TARBP*) supports the hypothesis that honeybee viruses are able to suppress the host RNAi machinery in order to infect and replicate within their host.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1** Virus state of the different samples used in this study.

**Table S2** Locus names, gene function or marker, oligo sequence and accession numbers for genes.

**Table S3** List of PCR primers used in this study for qPCR.

**Figure S1** Average expression stability (A) and determination of the optimal number (B) of reference targets with geNormPLUS.